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Rapid detection of *Salmonella* from hydrodynamic pressure-treated poultry using molecular beacon real-time PCR ☆

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Abstract

A real-time polymerase chain reaction (PCR) assay was evaluated to detect *Salmonella* in hydrodynamic pressure (HDP)-treated chicken using molecular beacon probes available as a commercial kit (iQ-Check, Bio-Rad Laboratories). The sensitivity and accuracy of the assay were compared with the conventional USDA microbiological procedure using artificially contaminated minced chicken. Chicken fillets were irradiated at 10 kGy to completely destroy any naturally occurring *Salmonella.* These fillets were minced and inoculated with as low as 2 ± 1 cfu of *S. typhimurium* per 25 g chicken. The minced chicken samples were vacuum packed in multi-layer barrier bags, heat shrunk, and treated with HDP. Results showed that all inoculated samples ($n = 36$) were detected by the PCR assay and conventional USDA procedure. Similarly, all uninoculated controls ($n = 11$) were negative by both PCR assay and USDA procedure. As few as 2 ± 1 cfu could be detected from 25 g HDP-treated chicken following 16–18 h enrichment in buffered peptone water. Real-time PCR proved to be an effective method for *Salmonella* detection in HDP-treated chicken with high sensitivity and more importantly, a rapid and high-throughput detection in 18 h, compared to 3–8 days for the conventional microbiological methods. HDP treatment, which has been reported to reduce spoilage bacteria in various meats, was unable to kill pathogenic *Salmonella* in minced chicken.
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Keywords: *Salmonella*; Poultry; Real-time PCR; Hydrodynamic pressure

1. Introduction

Salmonellosis is one of the most frequent foodborne diseases, being an important public health problem in almost all industrialized countries (D'Aoust, 1997). *Salmonella* is responsible for more than 1 million outbreaks, resulting in 500+ deaths every year in the United States (Mead et al., 1999), and is the leading causative agent among nine foodborne bacterial pathogens resulting in 39% of 15,600 laboratory-diagnosed infections in 2003 (CDC, 2004). A majority of cases of human salmonellosis are due to the consumption of contaminated egg, poultry, pork, beef, and milk products.

Conventional culture methods used for the detection of *Salmonella* include non-selective preenrichment, followed by enrichment and plating on selective and differential agars. Suspect colonies are confirmed biochemically and serologically, possibly taking up to 7 days to complete the entire procedure. More recently, a number of rapid methods for detection of *Salmonella* in foods have been developed including immuno-assays, nucleic acid hybridization, and polymerase chain reaction (PCR) techniques (Li et al., 2000). PCR assays for the rapid, sensitive, and specific detection of *Salmonella*

☆The USDA neither guarantees nor warrants the standard of the product, and the used of the name by USDA implies no approval of the product to the exclusion of other products that may also be available.

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have targeted genes such as *invA* (Chen et al., 1997a, b; Oliveira et al., 2002; Eyigor et al., 2002; Eyigor and Carli, 2003; Hong et al., 2003), the 16S rRNA gene (Trkov and Avgustin, 2003; Hong et al., 2003; Lin et al., 2004), *agfA* (Doran et al., 1993), virulence associated plasmid (Rexach et al., 1994), *viaB* (Hashimoto et al., 1995), and *sefA* (Szabo and Mackey, 1999; Oliveira et al., 2002; Medici et al., 2003). Most PCR assays employ either visual scoring of the amplification product by ethidium bromide stained agarose gels or post-PCR hybridization-capture methods that are labor intensive, time-consuming, and difficult to automate.

To overcome some of these concerns, we recently modified a commercially available, Association of Official Analytical Chemists (AOAC)-approved, PCR-based method (Bailey, 1998) for real-time monitoring by including the fluorescent dye SYBR Green I (Bhagwat, 2003, 2004). Although this modification enabled simultaneous and rapid detection of three foodborne pathogens in a high-throughput format, the fluorogenic reporter dye lacked specificity for the desired target molecule. Therefore, post-PCR melting curve analysis of the amplified product was incorporated in the protocol, and spurious amplification products were easy to distinguish (Bhagwat, 2004). However, a new fluorogenic PCR-based format has been recently developed which utilizes an internal fluorogenic probe that is specific to the target gene (Chen et al., 2000; Hoorfar et al., 2000). During the PCR assay, the target gene is amplified and simultaneously recognized and monitored by the fluorescent probe moiety (Tyagi and Kramer, 1996).

There are two types of fluorogenic PCR-based detection methods. One of the assay utilizes the 5′ nuclease activity of *Taq* DNA polymerase to hydrolyse an internal fluorogenic probe for monitoring amplification of DNA target (referred to as TaqMan assay) (Chen et al., 1997a; Hoorfar et al., 2000; Rodriguez-Lazaro et al., 2003), while the other like iQ-Check system (Bio-Rad Laboratories, Hercules, CA, USA) utilizes a fluorogenic probe which has flanking GC-rich arm sequences complementary to one another (Tyagi and Kramer, 1996; Chen et al., 2000; Liming and Bhagwat, 2004) (referred as molecular beacon, MB; Fig. 1). In both types of real-time PCR probes, a fluorescent moiety is conjugated to one end of the sequence, and a quencher moiety is attached to the other end of the sequence. In the absence of target DNA sequences, the MB assumes a hairpin conformation, with the two arms hybridizing to each other, thus bringing the quencher into close proximity to the fluorophore (which results in no or low background fluorescence). When the target DNA is present, the sequence in the loop region hybridizes, the hairpin of the MB opens, and the

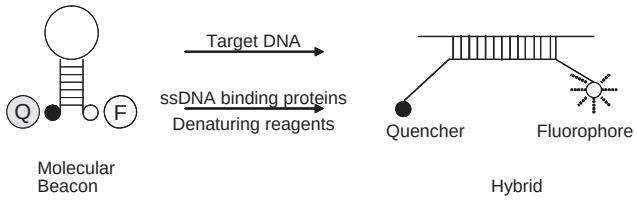

Fig. 1. MB structure showing the stem-loop conformation, which quenches fluorescence when the probe is alone in solution. In the presence of complementary target DNA or RNA, the beacon unfolds and hybridizes, and fluorescence is detected. MBs afford the user a closed-tube amplification detection and confirmation approach for real-time nucleic acid amplification.

fluorophore and the quencher separate. In the open conformation, the fluorophore of the MB emits a detectable signal that is directly correlated with the quantity of the target template present in the PCR assay (Tyagi and Kramer, 1996; McKillip and Drake, 2004). The TaqMan assay differs from the MB method in that the generation of the fluorophore signal is dependent upon 5′-3′-nuclease activity to cleave the reporter dye from the linear probe (Kimura et al., 1999; Nogva and Lillehaug, 1999).

Hydrodynamic pressure (HDP) processing is a relatively new, non-thermal process that was developed at the Food Technology and Safety Laboratory (Beltsville, MD, USA) for improving meat tenderness (Solomon, 1998). Supersonic–hydrodynamic shockwaves generated during HDP treatment disrupt the myofibrillar proteins of the meat muscle (Zuckerman and Solomon, 1998). HDP treatment was successfully utilized to significantly improve the tenderness of early deboned chicken breasts (Meek et al., 2000; Claus et al., 2001). Additionally, HDP treatment was also effective in reducing spoilage bacteria in ground beef and stew pieces (Williams-Campbell and Solomon, 2000, 2002). However, the effect of HDP in reducing pathogenic bacteria in meat is yet to be elucidated. Further, cells injured due to high pressure may remain viable but not culturable by rapid microbiological assays and therefore impart false-negative results (Chen et al., 1997a, b). Under favorable environmental conditions, these injured cells undergo repair, become functionally normal, and compromise the food safety. A rapid, sensitive assay for detection of very low levels of healthy or HDP-injured pathogenic *Salmonella* would be beneficial to the poultry industry. The objective of this study was to evaluate the use of a real-time, rapid, sensitive MB probe PCR assay in detecting *Salmonella* in HDP-treated chicken inoculated at the level of 2 cfu/25 g. The MB-PCR assay was compared with standard USDA microbiological procedure for correlation. Viability loss of pathogenic *Salmonella* in minced chicken following HDP treatment was reported.

2. Materials and methods

2.1. Bacterial strain and media

Salmonella typhimurium strain (MT 2195) lacking DNA adenine methylase (Dam^- mutant, Heithoff et al., 1999) was used in the study. This Dam^- mutant strain is non-pathogenic in nature which permitted us to carry out HDP treatment in open atmosphere using plastic explosive container (PEC). The strain was maintained in tryptic soy broth (TSB, Difco Laboratories, Detroit, MI, USA) containing 10% glycerol (Difco) and stored at −80 °C. Frozen cultures were partially thawed at room temperature (22–23 °C) for 15 min, streaked on Tryptic soy agar slants (Difco), and incubated at 37 °C for 24 h. Following overnight incubation, a loopful of growth was transferred to 10 ml LB Miller broth (Difco) containing 100 mM morpholinethanesulfonic acid (Amresco Inc., Solon, OH, USA), at pH 5.5 and incubated at 37 °C with shaking at 200 rpm for 20–22 h to obtain stationary-phase cultures (Chen et al., 2004). Cells obtained after at least two successive transfers as described above were resuspended in phosphate buffered saline (PBS, 50 mM) and adjusted to a cell density of $\sim 10^8$ cells ml^{-1} (1 o.d. at 600 nm) using a UV-2501 PC spectrophotometer (Shimadzu Corp., Japan). Cells were further diluted to obtain desired cell populations for inoculation into minced chicken. Cell concentrations were confirmed by spot plating on LB Miller agar (Difco).

2.2. Sample preparation and inoculation

Chicken breast fillets obtained from a local retailer were vacuumed packed and irradiated at 10 kGy (Food Technology Service Inc., Mulberry, FL, USA) to inactivate any indigenous *Salmonella* and stored frozen at −20 °C. Total microbial populations of irradiated chicken prior to *Salmonella* inoculation were non-detectable. Fillets (500 g) were thawed overnight and minced with a sterile knife. Minced chicken (100 g) was inoculated with as few as 10 *Salmonella* cells from serially diluted bacterial cultures (200-μl PBS). Higher inoculation levels ($\sim 2 \log_{10}$ cfu g^{-1}) were also used to determine the reduction in *Salmonella* populations, if any, following the HDP treatment. Each experiment was repeated four times with two samples per treatment.

2.3. Hydrodynamic pressure processing

Samples for HDP treatment were vacuum packed in Cryovac B620T bags (Sealed air Corp.). The sealed bag containing the samples was vacuum packed again in a multi-layer barrier bag (Cryovac BH620T bags, Sealed Air Corp., Duncan, SC, USA), and heat shrunk in hot water (88 °C) for 1–2 s. HDP was performed in a suspended 98-l PEC using 50 g binary explosive that was immersed in the water 30.5 cm away from the surface of the chicken samples. A 1.3 cm thick flat steel reflecting plate was used to support the chicken samples on the bottom of the PEC. To create an air boundary around the PEC, it was suspended 25 cm above the floor.

2.4. Conventional procedure

The USDA protocol for isolation and identification of *Salmonella* from meat, poultry, and egg products (USDA, 2002) was followed. Briefly, a 25 g chicken sample was placed into a sterile stomacher bag containing a filter (Microbiology International, Frederick, MD, USA) and diluted with 225 ml buffered peptone water (BPW, Difco, MI, USA), using an automatic diluter (Dilumat 3, AES Laboratoire, France) and pummeled for 2 min with a BagMixer® (Interscience, France). After preenrichment for 20–24 h at 35 °C, 0.5 and 0.1 ml of the preenriched samples were transferred to 10 ml tetrathionate broth (TT Hajna, Difco, MI, USA) and 10 ml Rappaport-Vassuliadis R10 broth (Difco), respectively. Broth tubes were incubated at 42 °C for 22–24 h, then streaked on Brilliant Green Sulfa (BGS, Difco) and Xylose Lysin Tergitol 4 agar (XLT4, Difco, MI, USA). Plates were incubated at 35 °C for 18–24 h. The identities of suspect *Salmonella* colonies on BGS and XLT4 agar were confirmed by the Salmonella Latex Test (Oxoid Ltd., UK).

To determine the lethal effect of HDP on *Salmonella* in chicken, samples inoculated with $\sim 2 \log_{10}$ cfu g^{-1} were immediately analysed following HDP treatment. Chicken samples (25 g) were mixed with an equal volume of 0.1% peptone water, pummeled for 2 min in a BagMixer® (Interscience), and spread plated on XLT4 agar (Difco). Bacterial colonies with characteristic *Salmonella* morphology were enumerated after an incubation of 24 h at 35 °C.

2.5. Cell lysis and PCR preparation

Chicken samples (25 g) were enriched in BPW for 18 h at 37 °C as recommended by the manufacturer of the iQ-Check *Salmonella* kit (Bio-Rad Laboratories, Hercules, CA, USA). Following incubation, 1 ml was withdrawn from the top layer without disturbing the food debris and centrifuged at 12,000 rpm (18,500*g*) for 5 min. After discarding the supernatant, the pellet was suspended in 200-μl lysis buffer (Bio-Rad) and vortexed. Lysis was carried out by incubating the suspension at 100 °C for 15 min in a dry-block heater (VWR International Inc., Bridgeport, NJ, USA).

DNA from pure cultures were prepared by mixing serially diluted 24 h cultures of *S. typhimurium* MT 2195 (10 μl) containing known quantities of viable cells

(measured as cfu ml^{-1}) with 90-μl lysis buffer (Bio-Rad). Four sets of *Salmonella* culture dilutions varying from 2.2 log_{10} cfu to 6.2 log_{10} cfu ml^{-1}, corresponding to 1–8200 cfu PCR^{-1} reaction were used. Lysis was carried out as described above and used as a standard in PCR assays.

2.6. PCR conditions and amplified product detection

Lysed samples were vortexed and centrifuged at 12,000 rpm (18,500*g*) for 5 min. For each sample, 5 μl of initial and 1:10 diluted DNA was mixed with 40 μl of amplification mixture and 5 μl of fluorogenic oligonucleotide MB probe labeled with FAM at the 5′-end and DABCYL at the 3′-end as the quencher. The fluorogenic MB probe from iQ-Check™ *Salmonella* kit (Bio-Rad Laboratories) targets the *iagA* (invasion associated gene) which is highly specific to *Salmonella* species (Miras et al., 1995). To monitor for successful DNA amplification in each reaction tube, kit provides a synthetic DNA as a part of the reaction mixture which works as an 'internal control'. This control was amplified with a specific probe at the same time as the *Salmonella* DNA sequence, and detected by a second fluorophore (Texas Red). The thermocycler (iCycler, Bio-Rad Laboratories) was programmed for 50 °C for 2 min, 95 °C for 5 min, 95 °C for 20 s, 55 °C for 30 s, 72 °C for 30 s) × 50 cycles, and 72 °C for 5 min. Data were collected after each annealing step using an excitation wavelength of 490 nm and an emission wavelength of 530 nm as specified by the manufacturer (Bio-Rad).

3. Results

3.1. Effect of HDP on Salmonella reduction in minced chicken

Salmonella populations survived in minced chicken following HDP treatment are shown in Table 1. Initial populations in minced chicken were in the range of 1.98–2.22 log_{10} cfu g^{-1} which were reduced to 1.70–1.95 log_{10} cfu g^{-1} following HDP treatment. The *Salmonella* reduction after HDP treatment was marginal (0.2–0.3 log_{10} cfu) and practically insignificant.

Table 1
Effect of HDP treatment on the survival of *S. typhimurium* inoculated in minced chicken[a]

Treatment	Colony counts (log_{10} cfu g^{-1})[b]		
	Rep 1	Rep 2	Rep 3
Control	1.98±0.10	2.22±0.11	2.02±0.12
HDP	1.70±0.26	1.95±0.11	1.81±0.10

HDP—hydrodynamic pressure treatment.
[a]A 100 g minced chicken inoculated with ~4 log_{10} cfu.
[b]Average and standard deviation of *Salmonella* counts obtained by plating on XLT4 agar.

3.2. Sensitivity of Salmonella specific PCR assay

We examined the sensitivity of a real-time MB-PCR method using dilutions of 24 h pure cultures of *S. typhimurium* MT 2195. Fig. 2 shows the relationship between cell populations and the efficiency of detection (measured as threshold cycle value, Ct). Ct values can be defined as the cycle at which a significant increase in fluorescence is first recorded. The Ct value increased as the number of the available template DNA molecules decreased. The figure shows a strong linear relationship between the log number of cells and Ct values with a correlation coefficient of 0.94. As low as 1 cfu of *Salmonella* per PCR reaction was detected using the assay.

3.3. Detection of Salmonella in HDP-treated chicken

The sensitivity of real-time iQ-Check™ PCR for detecting very low number of *Salmonella* in HDP-treated minced chicken was studied. Fig. 3 shows the normalized fluorescence measurements from the MB probe values (relative fluorescence units, RFU) versus PCR cycle collected in real time. Fluorescence from the MB probe (RFU) increased as the target DNA (*iagA*) accumulated at the end of each successive round of amplification. Data collected during the 30-s annealing cycle were used to quantify target DNA amplification. In artificially contaminated chicken samples, Ct value increased as the initial number of the template DNA molecules decreased, similar to the pure cultures. Thus, Ct values could be used to quantify target DNA molecules and subsequently cell populations in the PCR reaction mix.

The MB real-time PCR assay and conventional USDA procedure were performed in parallel for each

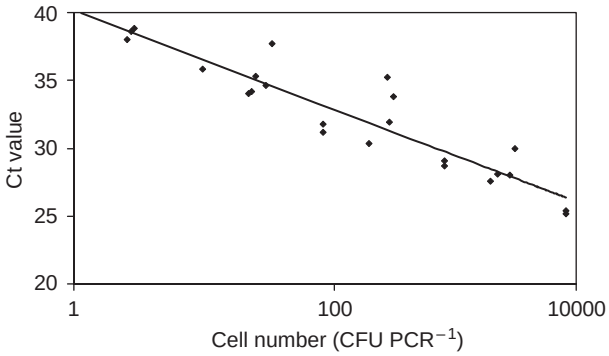

Fig. 2. Cell number (cfu PCR^{-1}) standard curve for the serially diluted pure culture of *S. typhimurium* determined by the real-time PCR assay Ct value is defined as the cycle at which a significant increase in the fluorescence is first recorded.

sample. Uninoculated chicken samples served as controls. There was no difference in Ct values of untreated and HDP-treated chicken samples (18.92 ± 1.66 and 20.20 ± 2.54, respectively) inoculated at 2 ± 1 cfu/25 g. Similar results were found at other inoculation levels (Table 2). Both protocols were able to detect *S. typhimurium* at low levels of inoculations (2 ± 1 cfu in 25 g chicken) in all samples tested. However, it took 3–5 days to obtain confirmatory results using the conventional USDA protocol compared to 18 h using the PCR assay.

4. Discussion

4.1. Effect of HDP treatment on Salmonella in chicken

The *Salmonella* reduction in minced chicken following the HDP treatment was 0.2–0.3 $\log_{10}$ cfu. It seems that lethal effect of HDP treatment on spoilage or pathogenic bacteria in food is not adequately defined. We investigated the efficacy of HDP treatment to reduce pathogenic *Salmonella* in minced chicken as the treatment has shown potential to significantly reduce the total plate counts in fresh ground beef (Williams-Campbell and Solomon, 2002) and intact beef muscle (Schilling et al., 2003). However, Lorca et al. (2002) did not find significant differences in coliform or *Escherichia coli* populations in ground beef following HDP treatment generated by electric arc.

Hydrostatic pressure processing (HPP) is another technology which utilizes very high pressure for cold pasteurization of foods. The HPP technology is being studied due to its ability to kill and injure microbial cells in various foods (Farkas and Hoover, 2000; Ray, 2002). Yuste et al. (2003) reported 3 $\log_{10}$ reduction of *S. typhimurium* in minced chicken following HPP treatment at 400 MPa for 1 min; however, the reduction was not significant (0.2 $\log_{10}$ cfu) when treated at 200 MPa for 5 min. The shock waves generated during HDP treatments are much lower (70–100 MPa) for fractions of milliseconds (Williams-Campbell and Solomon, 2002). Therefore, the shock waves may not be strong enough in the magnitude and the duration to have lethal effect on *S. typhimurium*.

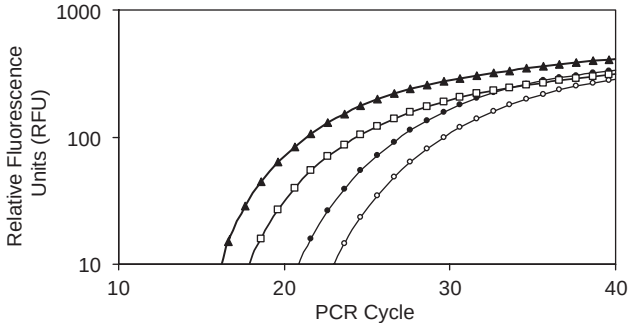

Fig. 3. PCR cycle real-time detection of *S. typhimurium* by measuring the fluorescence of FAM dye after each amplification cycle. RFU plotted for 18 h enriched BPW (cells per PCR assay as determined by standard curve 3.5 ○; 4.0 ●; 4.5 □; and 5.0 ▲ $\log_{10}$ cfu).

4.2. Real-time assay sensitivity

Application of PCR for the detection of pathogens in food samples is often limited by the presence of substances that inhibit the PCR reaction, poor quality of target DNA, or insufficient enrichment of target DNA (Rossen et al., 1992; Chen et al., 1997a; Heller et al., 2003). Chen et al. (1997a) suggested another centrifugation step of template DNA just prior to use in PCR to better remove PCR inhibitors and fluorescent contaminants. More cell debris and protein particles could be further removed from raw milk and chicken carcass rinse samples using additional centrifugation step. Soumet et al. (1997) suggested an enrichment step

Table 2
Evaluation of *S. typhimurium* detection in artificially contaminated chicken by real-time PCR method

Inoculation (cfu 25 g^{-1})	Treatment	Detection frequency[a]		
		Conventional USDA protocol	MB real-time PCR	
			iQ-Check	Ct value[a]
None	Control	0/11	0/11	NA
2	Control	8/8	8/8	18.92 ± 1.66
2	HDP	4/4	4/4	20.20 ± 2.54
5	Control	8/8	8/8	19.02 ± 2.08
5	HDP	4/4	4/4	19.55 ± 2.46
7	Control	8/8	8/8	19.36 ± 1.60
7	HDP	4/4	4/4	19.28 ± 1.85

NA—not applicable.
[a]Ct value is defined as the cycle at which a significant increase in the fluorescence is first recorded.

or an immuno-magnetic separation with additional enrichment to overcome false-negative results of *Salmonella* detection in chicken products. However, immuno-magnetic concentration of preenriched chicken samples was not advantageous in purifying *Salmonella* DNA (Medici et al., 2003). We followed the procedure described by Liming and Bhagwat (2004), who recommended enrichment in BPW and taking samples without disturbing the media at the end of the enrichment to keep the inhibition minimum. The PCR-ELISA assay for *Salmonella* yielded 5.0% false positives and 8.3% false negatives based on the assumption that the culture method is the standard for detection (Hong et al., 2003). Kurowski et al. (2002) reported 100% relative sensitivity with culture methods using multiple probes in PCR assay; however, the detection limit in the fecal sample was 230 organisms. Our MB-PCR assay had 100% agreement with the conventional USDA procedure with 11 negative (uninoculated) and 36 positive samples.

To date, culture techniques are universally recognized as the standard methods for the detection of bacterial pathogens such as *Salmonella* in foodstuffs. In theory, these methods are capable of detecting as few as one viable cell in a food sample following pre- and selective enrichment stages. Increased sensitivity of PCR assays has been reported for the detection of *Salmonella,* and is attributed to the fact that PCR can detect target sequences, irrespective of the growth potential of target cells (Chen et al., 1997a; Bailey, 1998; Whyte et al., 2002; Eyigor and Carli, 2003). PCR assay is usually more sensitive than the culture method because: (1) *Salmonella* with atypical biochemical profiles may not detected by culture methods but detectable by PCR assay; (2) lethally injured or dead cells may not be culturable but detected by PCR assay; and (3) growth of target cells may be prevented by the presence of other bacteria that can compete with *Salmonella* during preenrichment.

4.3. Detection limits

The detection sensitivity of real-time PCR to detect *Salmonella* in artificially inoculated poultry samples was 6 cfu ml^{-1} (Eyigor et al., 2002); however, the researchers used a selective enrichment step that could have affected the recovery of injured cells. Chen et al. (1997a) were able to detect 3 cfu in 25 g food samples using a fluorogenic *Taq*Man^{TM} probe. In their study, PCR assay detected more positive samples than the modified semi-solid Rappaport-Vassiliadis (MSRV) culture method. This may be due to the inability of injured cells to grow in MSRV medium or presence of a non-motile *Salmonella* strain not detected by MSRV method. Whyte et al. (2002) were able to detect as few as 10 cells without a DNA extraction step. Lin et al. (2004) tried to shorten the enrichment time so that the test could be completed in 1 day. While they were able to detect *Salmonella* in inoculated chicken meat after 8 h of enrichment, the sensitivity of the assay was 1 cfu g^{-1}. With the MB-PCR procedure, we were able to detect 2 cfu in 25 g chicken samples following 18 h preenrichment. Lack of background flora in irradiated chicken might have contributed to the increased PCR sensitivity in our study.

Bacterial pathogens are expected to be injured or inactivated by high-pressure treatment, depending on the pressure levels, species, and strain of the microorganism. Major injury, where the pathogen could form colonies on non-selective medium but not on selective medium (Bozoglu et al., 2004) could give false-negative results. To permit the recovery of HDP-treated injured cells, if any, we used a preenrichment step with BPW (USDA, 2002) that could allow the repair of any injured cells and subsequent detection by the PCR assay. The MB-PCR assay could be used as a valuable tool in screening the large number of samples for *Salmonella* in poultry.

Acknowledgements

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